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# Neuronal and Nonneuronal Quantitative BACE Immunocytochemical Expression in the Entorhinohippocampal and Frontal Regions in Alzheimer's Disease

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**Key Words**
Quantitative immunocytochemistry · β-Site amyloid precursor protein cleaving enzyme · Amyloid · Neurons · Astrocytes · Senile plaques · Neurofibrillary tangles · Entorhinal cortex · Frontal cortex · Hippocampus

**Abstract**
In this study, we quantitatively investigated the expression of β-site amyloid precursor protein cleaving enzyme (BACE) in the entorhinohippocampal and frontal cortex of Alzheimer's disease (AD) and old control subjects. The semiquantitative estimation indicated that the intensity of BACE overall immunoreactivity did not differ significantly between AD and controls, but that a significantly stronger staining was observed in the hippocampal regions CA3–4 compared to other regions in both AD patients and controls. The quantitative estimation confirmed that the number of BACE-positive neuronal profiles was not significantly decreased in AD. However, some degeneration of BACE-positive profiles was attested by the colocalization of neurons expressing BACE and exhibiting neurofibrillary tangles (NFT), as well as by a decrease in the surface area of BACE-positive profiles. In addition, BACE immunocytochemical expression was observed in and around senile plaques (SP), as well as in reactive astrocytes. BACE-immunoreactive astrocytes were localized in the vicinity or close to the plaques and their number was significantly increased in AD entorhinal cortex. The higher amount of β-amyloid SP and NFT in AD was not correlated with an increase in BACE immunoreactivity. Taken together, these data accent that AD progression does not require an increased neuronal BACE protein level, but suggest an active role of BACE in immunoreactive astrocytes. Moreover, the strong expression in controls and regions less vulnerable to AD puts forward the probable existence of alternate BACE functions.



## Introduction

Alzheimer's disease (AD) is characterized by the extracellular deposition of a 4-kDa peptide of 40–42 aa, the β-amyloid peptide (Aβ). Aβ is deposited into senile plaques (SP) and the relationship between SP, neurofibrillary tangles (NFT) and the pathogenic mechanism of AD

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is controversial. However, several lines of evidence indicate that Aβ deposit is involved at an early stage of the disease. Aβ is cleaved from the ubiquitous amyloid precursor protein (APP), sequentially by two secretases, first the β-secretase – producing an ectodomain of APP named APPsβ and the C-terminal 99 amino acids of APP (C99) – then the γ-secretase, generating the C-terminus of Aβ. Mutations around the β- and γ-secretase cleavage site in APP lead to early-onset AD, probably due to an increased affinity of the secretases for the APP substrate [1].

The enzyme responsible for the main β-secretase cleavage has been identified and cloned. It corresponds to a membrane-bound aspartyl protease called β-site APP cleaving enzyme – BACE1 or BACE – also named Asp2 or Memapsin2 [2]. Another β-secretase, BACE2, has been isolated but the level of BACE2 mRNA is very low or almost undetectable in human and rat brain while higher in peripheral tissues [3]. It has been shown that BACE is the major β-secretase for generation of Aβ in neurons [4] and would thus represent the key enzyme initiating the formation of Aβ in the brain. However, no mutations or significant polymorphisms in the sequence of BACE gene have been found until now in different populations of AD patients [5–9], although the combination of BACE-specific allele and ApoE4 may slightly increase the risk of AD above that of ApoE4 alone [10]. Nevertheless, inhibition of BACE activity represents an attractive drug target for AD [11], as knockout mice are healthy despite lacking the primary β-secretase activity in the brain [12]. The lack of Aβ generation in the brain of BACE-deficient mice overexpressing the Swedish APP mutation suggests that the therapeutic BACE inhibition could reduce Aβ in the brain of AD patients [11].

The few studies on the BACE protein level, BACE mRNA and BACE enzymatic activity in human AD patients generally show an increase in BACE protein expression in the cerebral cortex of AD patients compared to controls [13, 14], an increase [14] or stable BACE transcription [15, 16], and an increase in BACE enzymatic activity in various areas and to various degrees [14, 17, 18]. However, only one paper studied the distribution and localization of BACE by immunocytochemical methods in the entorhinal cortex (EC) and hippocampus. It reveals both an increase in the CA1 region and a decrease in the EC [19].

While BACE inhibition is a promising drug target, its immunocytochemical expression needs to be further investigated with quantitative methods. Our study is the first presenting both a semiquantitative analysis of BACE immunoreactivity related to the amount of SP and NFT in the hippocampus, the subiculum (SUB) and the EC, but also in the frontal cortex (FC), together with a quantitative study of BACE immunopositive profiles.

## Materials and Methods

*Brain Samples*

Brain samples were collected from 12 AD patients, both sporadic AD and familial AD (FAD) (mean age: 77.5 years), and 16 age-matched controls (mean age: 72.8 years) without neurological disease (table 1). Brains were fixed in 10–15% formalin for 4 weeks and stored in 5% formalin. Tissue blocks were taken from the EC and from the hippocampal formation including dentate gyrus (DG) and regions 1–4 of Ammon's horn (CA1–4), as well as from the frontal cortex area 9 (FC9) or 10 (FC10) of Brodmann. Not all regions were available in all cases (table 1). Blocks were embedded in paraffin and serial 7-μm-thick sections were generated. Alternate sections were used for BACE, Aβ and glial fibrillary acidic protein (GFAP) immunocytochemistry, and for the Gallyas silver iodide [20] method to detect NFT degeneration. Double immunocytochemical staining of BACE/Aβ and of GFAP/Aβ was also performed. Finally, a two-step protocol was used to visualize BACE immunocytochemistry and Gallyas staining on the same section.

*Immunocytochemical Methods*

BACE Immunocytochemical Staining

BACE immunocytochemical staining was performed using a polyclonal goat anti-BACE IgG raised against the human BACE1-specific amino acid sequence 485–501 (Chemicon International Inc. AB5488, dilution 1:2,500; no similarity to BACE2). A biotinylated rabbit antigoat (Dako Diagnostics AG E0466, dilution 1:300) was used as secondary antibody. The specificity of the BACE antibody is guarantied by the firm for formalin-fixed paraffin-embedded sections and has been demonstrated both with Western blots and immunocytochemistry [21]. Immunocytochemistry also employed an avidin-biotin-peroxidase complex kit (ABC kit; Dako K0355; solutions A and B, dilution 1%) and normal rabbit serum (Dako X0902, dilution 1:10). Phosphate-buffered saline (PBS; 0.01 *M* and 0.1 *M*, pH 7.4) was used for rinsing and for dilution of 3,3′-diaminobenzidine (DAB; Sigma Aldrich Chemie D5637), which was employed for antibody revelation. PBSA (1% of albumin from bovine serum in PBS, 0.01 *M*, pH 7.4) was used for antibodies and ABC kit dilution.

Deparaffinized sections were permeabilized with 0.1% Triton X-100 in PBS 0.1 *M* for 15 min, rinsed 3 times in PBS 0.1 *M*. To reduce background, they were pretreated in 10% methanol and 0.3% $H_2O_2$ (30%) in PBS 0.1 *M* for 15 min and rinsed twice in water. To enhance immunocytochemical staining, they were put in buffered citric acid (0.01 *M*; pH 6.0) in the microwave (800 W) for 5 min. After rinsing twice in PBS 0.01 *M* (2 × 2.5 min) and incubating for 10 min in normal rabbit serum (dilution 1:10), incubation with the first antibody (dilution 1:2,500) was carried out overnight at 4°C. After rinsing in PBS 0.01 *M* (2 × 5 min), sections were incubated with the secondary antibody (dilution 1:300) for 1 h at room temperature. Sections were rinsed twice in PBS 0.01 *M* and incubated in a solution of ABC kit for 35 min at room temperature. After rinsing twice in PBS 0.01 *M*, staining was revealed for 10 min in 0.03% DAB and 0.015% $H_2O_2$ in PBS 0.01 *M*. Sections were rinsed twice in tap

**Table 1.** Description of cases and availability of material

| Case | Sex | Age | PMD, h | Group | Cause of death | Region |
|---|---|---|---|---|---|---|
| 1 | M | 30 | 12 | C | myocardial infarct | FC10 |
| 2 | M | 48 | 10 | C | myocardial infarct | Hipp/FC10 |
| 3 | M | 52 | 16 | C | myocardial infarct | FC10 |
| 4 | M | 55 | 19 | C | cardiac failure | FC10 |
| 5 | M | 71 | 38 | C | pancreatic carcinoma | FC10 |
| 6 | F | 71 | 11 | C | cardiac insufficiency | FC10 |
| 7 | F | 77 | 19 | C | pulmonary embolism | Hipp |
| 8 | M | 78 | 22 | C | urinary bladder carcinoma | Hipp/FC9 |
| 9 | M | 78 | 28 | C | bronchopneumonia | Hipp/FC9 |
| 10 | M | 78 | 24 | C | thrombosis | FC10 |
| 11 | M | 84 | <24 | C | cachexia | FC10 |
| 12 | F | 84 | 30 | C | myocardial infarct | Hipp/FC9 |
| 13 | F | 87 | 92 | C | uterus carcinoma | Hipp |
| 14 | M | 89 | 15 | C | bronchopneumonia | FC9 |
| 15 | F | 90 | 61 | C | myocardial infarct | Hipp |
| 16 | M | 93 | 18 | C | infection | FC10 |
| 17 | M | 55 | 18 | FAD | coma | FC10 |
| 18 | M | 67 | 1 | AD | bronchopneumonia | FC10 |
| 19 | M | 69 | 9 | FAD | cardiac failure | Hipp/FC10 |
| 20 | M | 71 | 20 | FAD | breathing failure | Hipp/FC10 |
| 21 | M | 71 | 20 | AD | cachexia | FC10 |
| 22 | F | 80 | 21 | AD | renal insufficiency | Hipp/FC9 |
| 23 | F | 80 | – | AD | – | FC10 |
| 24 | M | 81 | 28 | AD | bronchoaspiration | FC10 |
| 25 | M | 86 | 23 | AD | bronchopneumonia | FC10 |
| 26 | F | 87 | 64 | AD | cardiac insufficiency | Hipp/FC9 |
| 27 | F | 88 | 7 | AD | cardiac insufficiency | Hipp/FC9 |
| 28 | F | 95 | 16 | AD | myocardial infarct | Hipp/FC9 |

M = Male; F = female; C = control case; AD = Alzheimer's disease; FAD = familial Alzheimer's disease; PMD = postmortem delay; Hipp = hippocampal region including EC and SUB; FC9 = frontal area 9; FC10 = frontal area 10.

water, twice in water and counterstained with Mayer hematoxylin without citric acid for 1 min, then rinsed tree times in tap water and counterstained in a Scott blue solution for 30 s. Sections were dehydrated and mounted with Pertex. For each batch of sections, control consisted of omitting primary antibody.

Aβ and GFAP Immunocytochemical Staining

Aβ immunocytochemical staining was performed using a monoclonal mouse anti-Aβ IgG raised against the human sequence 8–17 of the Aβ protein (Dako M0872, dilution 1:100) and a biotinylated rabbit antimouse (Dako E0413, dilution 1:300) as secondary antibody. Immunocytochemical staining of GFAP (principal intermediate filament of astrocytes) was detected using a polyclonal rabbit antihuman GFAP (Dako Z0334, dilution 1:1,500) and a biotinylated swine antirabbit immunoglobulin (Dako E0353, dilution 1:300) as secondary antibody. All sections were pretreated in methanol: $H_2O_2$ (97:3) for 10 min to reduce background, rinsed twice in water and only Aβ sections were put in 80% formic acid for 5 min to enhance immunocytochemical staining. After rinsing twice in water and twice in PBS 0.01 *M*, sections were incubated for 10 min in normal rabbit serum (Aβ) or normal swine serum 1:10 (GFAP) and then with primary antibody over night at 4°C. After rinsing in PBS 0.01 *M* (2 × 5 min), sections were incubated with the secondary antibody for 1 h at room temperature. Revelation, counterstaining and control were performed as above.

BACE/Aβ and GFAP/Aβ Double Immunocytochemical Staining

To reveal BACE, a peroxidase substrate (Novared kit, Vector Laboratories, Inc. SK-4800) was used. Then a nickel method was used to reveal the Aβ labeling, employing a solution of Tris buffer (0.1 *M*; pH 7.4) made of 0.016 *M* Trizma base and of 0.084 *M* Trizma-HCl in water, solution A made of 0.2% $(NH_4)_2Ni(SO_4)_2 \times 6H_2O$ in Tris buffer, solution B made of 0.2% $(NH_4)_2Ni(SO_4)_2$ with 0.035% DAB in Tris buffer and solution C made of solution B plus 0.01% $H_2O_2$ (30%). Pretreatment was identical to pretreatment of the BACE method except for the citric acid bath replaced by the formic acid bath (due to the combination with Aβ immunocytochemical staining; see above) for 5 min at room temperature. To perform the first immunocytochemical staining, sections were incubated in normal rabbit serum (see above), then with the primary anti-BACE antibody at 4°C overnight, rinsed in PBS 0.01 *M* (2 × 5 min) and incubated with the secondary antibody (dilution 1:300) for 1 h at room temperature. Sections were rinsed twice in PBS 0.01 *M* and incubated in a solution of the ABC kit for 35 min at room temperature.

After rinsing twice in PBS 0.01 *M*, sections were revealed using the peroxidase substrate Novared kit for 10 min. To perform the secondary staining, sections were rinsed twice in tap water, twice in water, twice in PBS 0.01 *M* and incubated in normal rabbit serum for 10 min (see above). Sections were incubated at 4°C overnight with the primary anti-Aβ antibody (dilution 1:100), rinsed in PBS 0.01 *M* (2 × 5 min) and incubated with the secondary anti-Aβ antibody (dilution 1:300) for 1 h at room temperature. After rinsing twice in PBS 0.01 *M*, sections were incubated in an ABC kit for 35 min at room temperature. Sections were rinsed twice in PBS 0.01 *M* and the peroxidase was revealed by conditioning in Tris buffer (twice) for 10 min, in solution A for 10 min, in solution B for 10 min and in solution C for 10 min. Counterstaining was performed as above. GFAP/Aβ double immunocytochemical staining was identical to BACE/Aβ double immunocytochemical staining. GFAP was labeled first (antibody's dilution 1:2,500), revealed with the Novared kit and Aβ was labeled afterwards (antibody's dilution 1:100), revealed using the nickel method.

BACE/Gallyas Two-Step Protocol

BACE immunocytochemistry was performed first as described for BACE simple immunocytochemical staining and photographs were taken at specific locations on humid unmounted sections; modified Gallyas silver iodide staining [20] with light green SF (Chroma-Gesellschaft no 1B211) counterstaining was performed on the same section and photographs were taken at the same locations, allowing detection of possible signal colocalization.

*Semiquantitative Study and Statistical Analysis*

BACE immunopositive neurons, Aβ SP, NFT and mature GFAP-positive astrocytes were estimated semiquantitatively under bright-field illumination using a Zeiss Axioplan microscope, at a magnification of ×200, in all sampled regions, i.e. in all layers of EC, FC9 and FC10, as well as in DG, CA3–4 and CA1 regions of the hippocampus and in the SUB. The screening was performed by 2 independent people using at least 12 fields on 2–3 different sections in each region and in each brain. In addition, each observer performed screenings twice in order to check his own observations. Estimations were comparable, taking into account both the amount of stained elements and the intensity of the staining. Tables 3 and 4 indicate that they varied from 0 (no staining) to + (weak staining), ++ (medium staining), +++ (strong staining) and ++++ (very strong staining). For data analysis, these semiquantitative estimations were graded from 0 to 4 (fig. 2). We have recently used this method to quantify LMO4 protein immunoexpression in Alzheimer brains [22].

Data were analyzed with the help of a statistical analysis package SAS [23]. Explanatory data analysis included a box-plot representation of the estimations of BACE immunocytochemical staining, Aβ-stained SP, Gallyas-marked NFT and GFAP-reactive astrocytes (fig. 2). A nonparametric test such as Kruskal-Wallis variance analysis was used to compare groups and a Spearman correlation analysis was performed between parameters.

*Quantitative Study and Statistical Analysis*

In addition to the semiquantitative analysis, BACE immunopositive profiles were quantified with an image analysis system in a column of cortex from the EC and from the FC10.

Morphometry was performed using a Zeiss/Kontron image analysis system. This system has a stage with stepping motors for the 3 axes and a video camera for image capture (512 × 512 pixels). The histological section can be moved under program control in order to scan the whole cortical depth along columns going perpendicularly from pia to white matter. For the best acuity, each individual field was viewed at a magnification of ×125 for EC, which made the counting window 448 μm wide and 324 μm high, and at a magnification of ×250 for FC, which made the counting window 227 μm wide and 163 μm high. Three cortical columns were screened in EC and FC10, depending on the available material (table 1). We quantified the number of BACE well-marked neuronal profiles, corresponding to complete cells in the section, using a semiautomatic threshold procedure, based on the optical density of neuronal profiles. The optical density corresponded to the intensity of the transmitted light and was measured on a scale going from 0 (100% transmitted light) to 255 (0% transmitted light) for each pixel. The profile segmentation was performed in an interactive way, including the possibility of visual correction by the user (elimination of artifacts or separation of fused profiles). Criteria of object inclusion were the shape and the size of the neuronal perikarya, as well as a strong immunoreactive status. Parameters such as the position of the neuronal profile in the column and the neuronal surface area were measured by means of our specifically designed software. In parallel, we quantified the number of BACE-immunonegative neurons – including very weakly marked neurons – as well as the number of BACE-immunopositive typical reactive astrocytes. Knowing the surface of the cortical column, we calculated the number of cellular profiles per square millimeter of cortex (densities), in 7-μm-thick sections (table 4). Indeed, the number of profiles reflects the number of neurons in sections of similar thickness treated with similar procedures. Data were analyzed with the help of the statistical analysis package [23]. As the distributions of densities or surface areas of neuronal profiles did not always fulfill criteria for normality, differences in the densities of neuronal profiles between AD and controls were analyzed with a Kruskal-Wallis nonparametric test and the distribution of surface areas with a $\chi^2$ test.

## Results

### *BACE Protein Immunoreactivity in Control and AD Brains*

BACE immunostaining was present in the control brains in all regions examined (fig. 1A–F). It was mainly specific for the neuronal cell bodies and did not mark glial or endothelial cells except in some localizations, where reactive astrocytes with fibrillary prolongations could be observed. The neuronal staining appeared intracytoplasmic, making a ring around the nucleus and often at the basis of the dendrites, which was clearly shown by the blue counterstaining of the nucleus. In the SUB and in the EC, most neurons were marked, but the staining was particularly strong in EC layers 2 and 5. In the hippocampus, all subdivisions were marked including the DG, but the region CA3–4 exhibited the strongest staining, followed by the region CA1. In the FC, BACE staining was present in both available areas 9 and 10 and the intensity was qualitatively similar to that in other regions. Semiquanti-

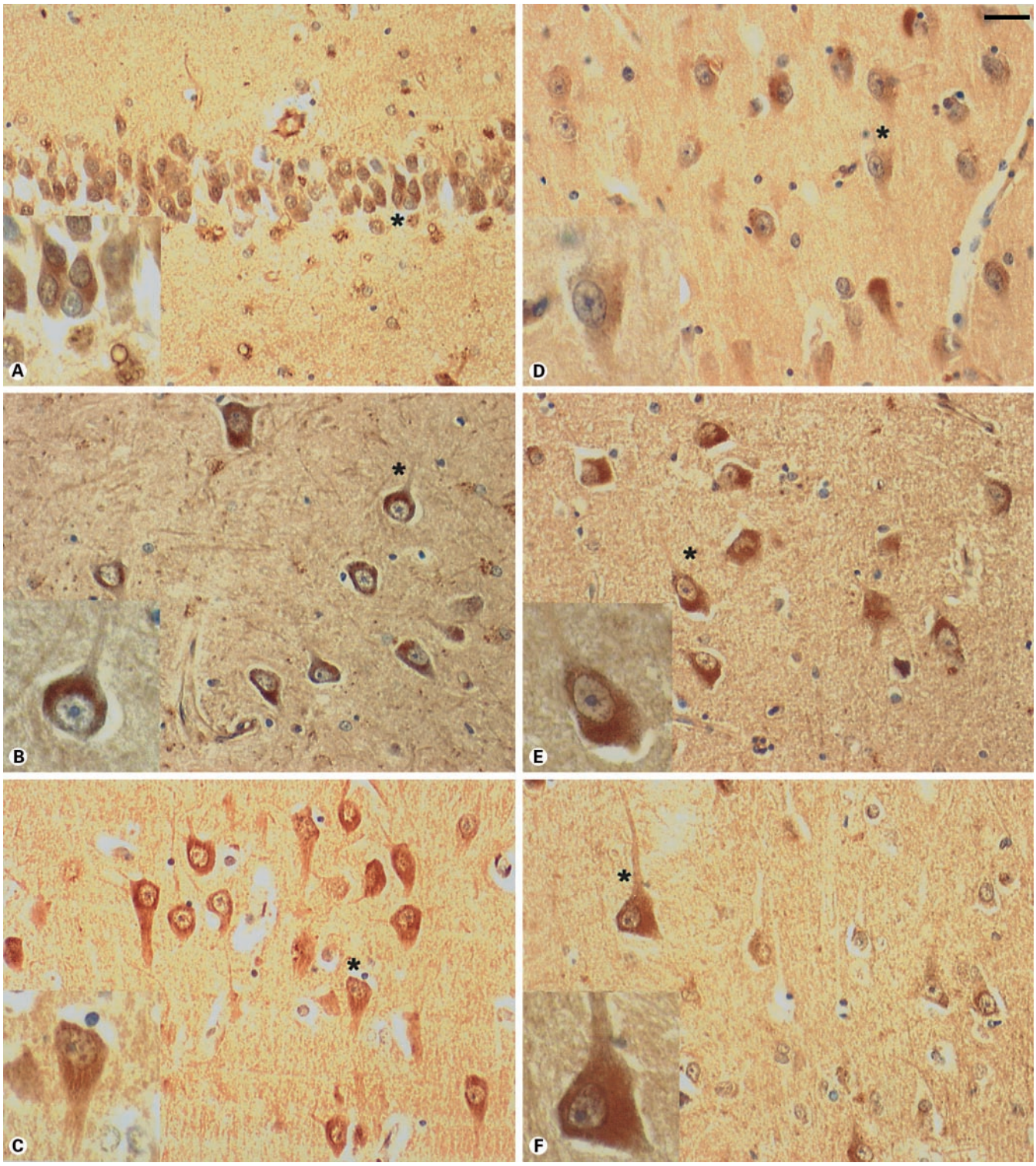



**Fig. 1.** Photomicrographs of 7-µm paraffin sections showing BACE immunocytochemical staining with anti-BACE (Chemicon AB5488, dilution 1:2,500) in 6 different regions of control brains. Counterstaining with Mayer hematoxylin and Scott blue. **A** Case No. 15 (table 1): DG. **B** Case No. 8: CA3–4. **C** Case No. 13: CA1. **D** Case No. 12: SUB. **E** Case No. 8: layer 2 of EC. **F** Case No. 8: layer 5 of FC; inserts show magnification of one neuron in each case (asterisk). Immunocytochemical staining – clearly cytoplasmic – is the strongest in the region CA3–4 (see also fig. 2). Scale: 50 µm; insert: 25 µm.

**Table 2.** Semiquantitative estimation of BACE immunostaining in the entorhinohippocampal region

| Case | Sex | Age | Group | DG | | | | CA3–4 | | | | CA1 | | | | SUB | | | | EC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | BACE | SP | NFT | GFAP | BACE | SP | NFT | GFAP | BACE | SP | NFT | GFAP | BACE | SP | NFT | GFAP | BACE | SP | NFT | GFAP |
| 2 | M | 48 | C | ++ | 0 | 0 | 0 | ++++ | 0 | 0 | ++ | +++ | 0 | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | 0 | 0 |
| 7 | F | 77 | C | + | 0 | 0 | 0 | +++ | 0 | 0 | ++ | ++ | 0 | 0 | 0 | + | 0 | 0 | 0 | +++ | 0 | 0 | 0 |
| 8 | M | 78 | C | ++ | 0 | 0 | 0 | ++++ | 0 | 0 | ++++ | ++ | 0 | 0 | 0 | ++ | 0 | 0 | 0 | ++ | 0 | 0 | 0 |
| 9 | M | 78 | C | ++ | 0 | 0 | 0 | +++ | 0 | 0 | ++++ | 0 | 0 | 0 | + | 0 | 0 | 0 | 0 | ++ | 0 | + | ++ |
| 12 | F | 84 | C | ++ | 0 | 0 | 0 | ++++ | 0 | 0 | ++++ | +++ | 0 | + | 0 | ++ | 0 | + | 0 | ++ | + | +++ | + |
| 13 | F | 87 | C | ++ | 0 | 0 | 0 | +++ | 0 | 0 | +++ | +++ | 0 | 0 | 0 | +++ | 0 | 0 | 0 | +++ | 0 | ++ | 0 |
| 15 | F | 90 | C | ++ | 0 | 0 | 0 | ++++ | 0 | 0 | +++ | ++++ | 0 | 0 | 0 | ++ | 0 | 0 | 0 | +++ | 0 | + | 0 |
| 19 | M | 69 | FAD | ++ | + | 0 | 0 | ++++ | ++ | + | +++ | ++ | ++ | ++ | 0 | ++ | 0 | ++++ | ++ | ++ | + | +++ | ++ |
| 20 | M | 71 | FAD | 0 | 0 | 0 | 0 | + | ++ | ++ | ++++ | ++ | 0 | + | ++++ | + | ++ | +++ | ++++ | 0 | ++ | +++ | ++++ |
| 22 | F | 80 | AD | ++ | 0 | 0 | 0 | ++++ | 0 | + | ++++ | +++ | + | +++ | 0 | ++ | 0 | ++++ | + | ++ | ++ | +++ | ++ |
| 26 | F | 87 | AD | ++ | 0 | 0 | 0 | +++ | 0 | 0 | ++++ | ++ | 0 | ++ | ++ | ++ | 0 | ++++ | +++ | ++ | ++ | ++ | ++ |
| 27 | F | 88 | AD | + | 0 | 0 | 0 | +++ | + | 0 | +++ | +++ | + | 0 | 0 | +++ | ++ | ++ | 0 | ++ | +++ | ++ | + |
| 28 | F | 95 | AD | + | 0 | 0 | 0 | ++++ | 0 | 0 | +++ | ++ | + | ++ | ++ | ++ | ++ | ++++ | ++ | ++ | +++ | ++ | ++ |

M = Male; F = female; C = control case; BACE = immunostaining with the polyclonal antibody goat anti-BACE; SP = senile plaques immunostained with the monoclonal antibody mouse anti-Aβ; NFT = neurofibrillary tangles stained with the Gallyas method; GFAP = immunostaining with the polyclonal antibody rabbit antihuman glial fibrillary acidic protein. Semiquantitative estimation data, taking into account both the intensity of staining and the number of stained elements, were graded as 0 = no staining; + = weak; ++ = medium; +++ = strong; ++++ = very strong.

tative data from areas 9 and 10 were pooled (table 3; fig. 2)

The overall staining estimation, taking into account both the staining intensity and the number of stained elements, was not markedly different in control cases from different ages (tables 2, 3), meaning that aging alone does not affect the amount of intraneuronal BACE expression in the studied regions. Similarly, the semiquantitative estimation of BACE immunostaining revealed no marked differences between AD and control brains in any of the regions (tables 2, 3). Kruskal-Wallis analysis confirmed that there were no statistically significant differences between control and AD brains. In both controls and AD patients, greater BACE expression was found in the region CA3–4 compared to other regions (controls: $p = 0.001$; AD patients: $p = 0.03$). Thus, the global neuronal BACE expression did not change in AD, with, however, a trend to lower values for EC, where the probability ($p = 0.056$) was just at the limit of significance.

*BACE Protein Immunoreactivity in Relation to SP and NFT*

We observed – rarely in old controls, mainly in AD entorhinohippocampal regions – BACE expression in the SP as well as in reactive astrocytes (fig. 3A–C). Using either simple staining with the anti-BACE antibody (fig. 3A) or double staining with both anti-BACE and anti-Aβ antibodies (fig. 3B), we could observe that BACE expression was localized in the periphery of the plaque, while the center was strongly positive for Aβ. There were two different types of SP, with the periphery showing either diffuse BACE reactivity or intermingled Aβ and BACE reactivity. In addition, reactive BACE-positive astrocytes were also observed at the border of the plaque (fig. 3B). When using a GFAP antibody, similar features were observed, with strong GFAP staining at the periphery of many SP and GFAP-positive astrocytes at the border of SP (fig. 3C).

SP stained with anti-Aβ and NFT marked with Gallyas were estimated semiquantitatively on sections adjacent to BACE staining estimation (tables 2, 3; fig. 2). Kruskal-Wallis analysis indicated that the increase in SP estimation in AD compared to control cases was statistically significant in all regions except in DG (EC: $p = 0.003$; SUB: $p = 0.001$; CA1: $p = 0.001$; CA3–4: $p = 0.004$; FC: $p < 0.001$). The amount of NFT was also increased in a statistically significant way in all AD regions except in DG (EC: $p = 0.02$; SUB: $p = 0.002$; CA1: $p = 0.005$; CA3–4: $p = 0.001$; FC: $p < 0.001$). In addition, taking into account anatomical landmarks such as capillaries on two adjacent sections permitted to detect that BACE immunostaining and NFT degeneration often concerned similar neuronal populations. In several entorhinohippocampal localizations, but mainly in the SUB, neurons expressing BACE immunocytochemistry were also marked for NFT degeneration. Further, using a two-step procedure for double-labeling on the same section (see Materials and Methods) ascertained that they were exactly the same neurons

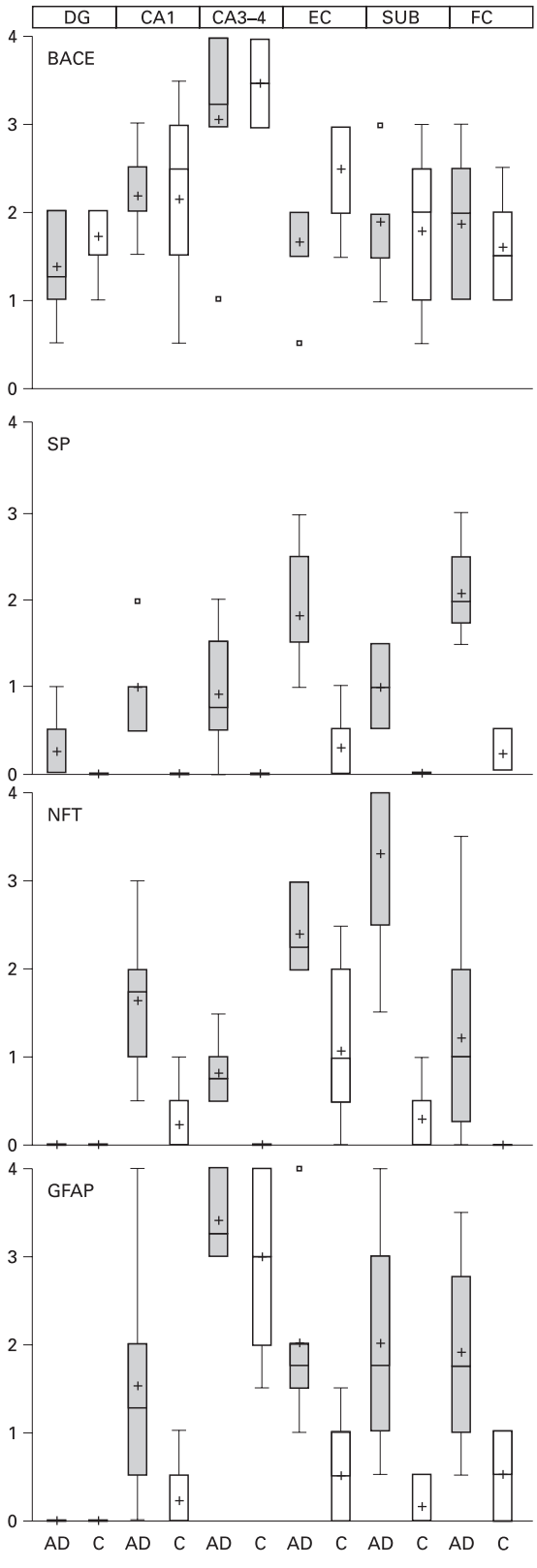
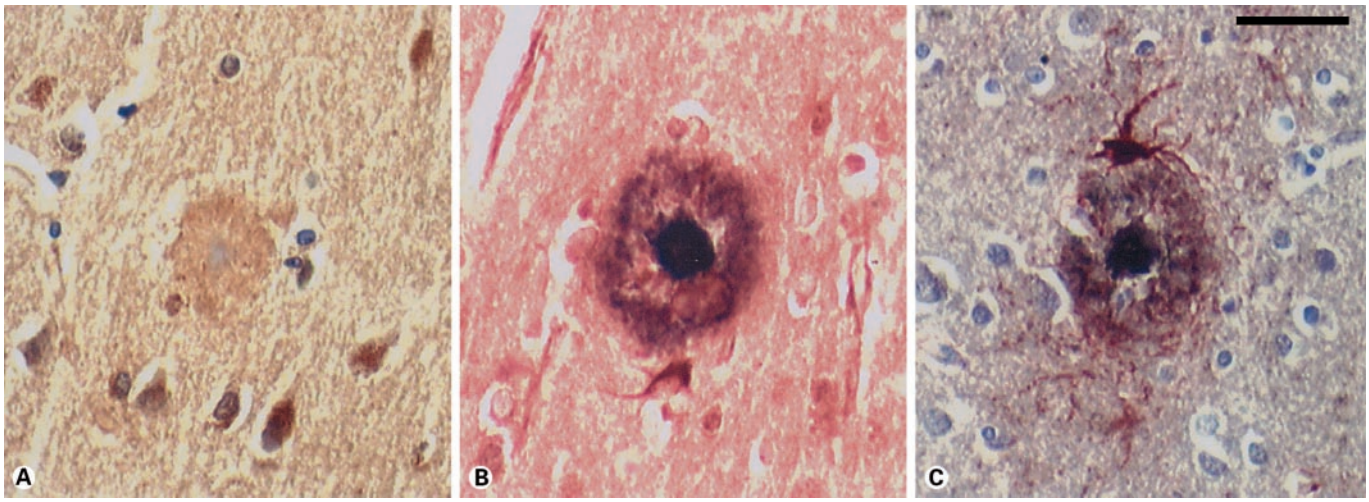



**Table 3.** Semiquantitative estimation of BACE immunostaining in the FC (areas 9 and 10)

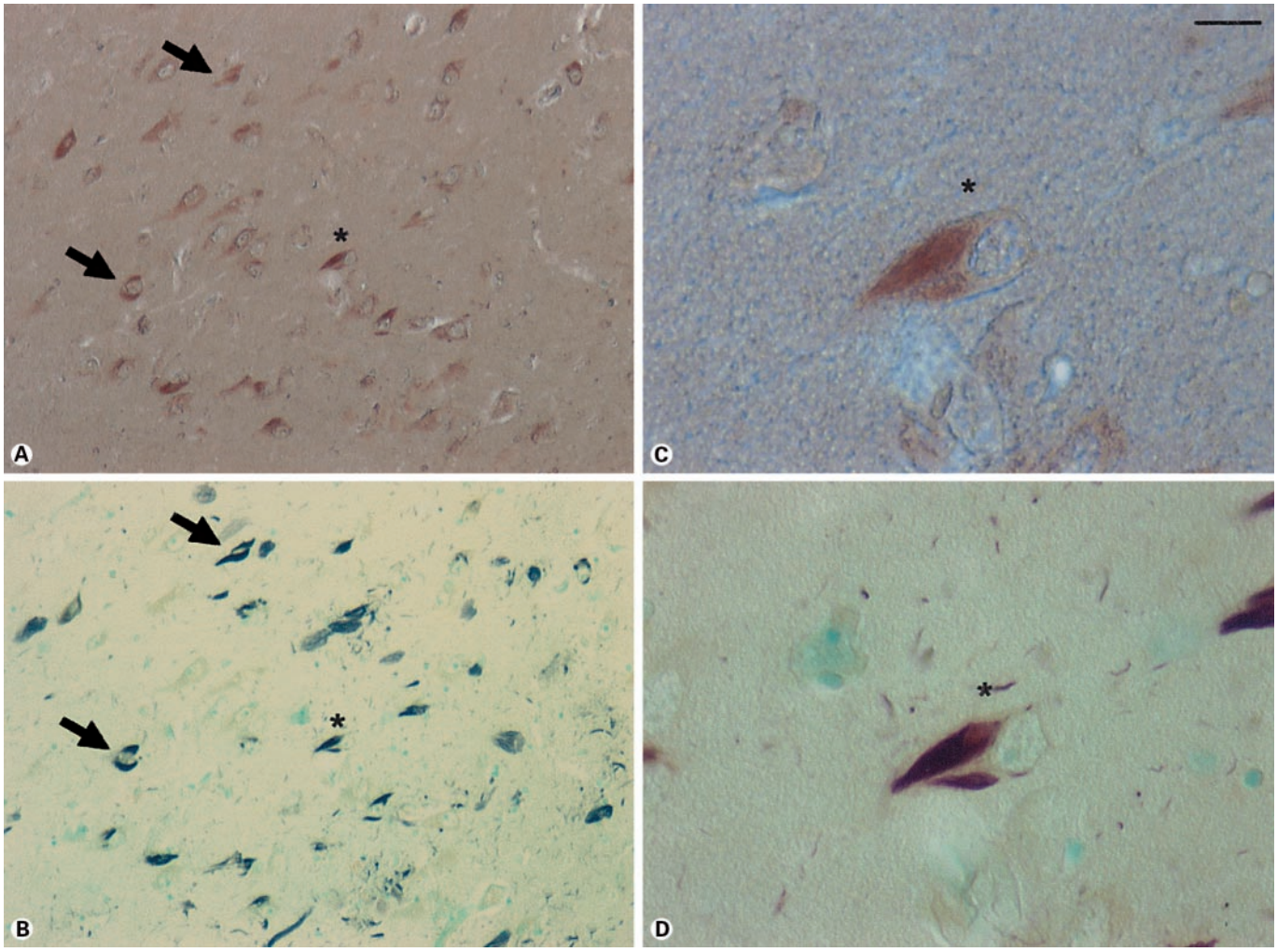
| Case | Sex | Age | Group | BACE | SP | NFT | GFAP |
|---|---|---|---|---|---|---|---|
| 1 | M | 30 | C | + | 0 | 0 | 0 |
| 2 | M | 48 | C | ++ | 0 | 0 | 0 |
| 3 | M | 52 | C | ++ | 0 | 0 | 0 |
| 4 | M | 55 | C | + | 0 | 0 | 0 |
| 5 | M | 71 | C | ++ | 0 | 0 | + |
| 6 | F | 71 | C | ++ | 0 | 0 | 0 |
| 8 | M | 78 | C | + | 0 | 0 | 0 |
| 9 | M | 78 | C | +++ | 0 | 0 | + |
| 10 | M | 78 | C | ++ | 0 | 0 | + |
| 11 | M | 84 | C | ++ | 0 | 0 | 0 |
| 12 | F | 84 | C | + | 0 | 0 | 0 |
| 14 | M | 89 | C | + | 0 | 0 | 0 |
| 16 | M | 93 | C | ++ | 0 | 0 | + |
| 17 | M | 55 | FAD | + | ++ | ++++ | ++++ |
| 18 | M | 67 | AD | +++ | ++ | + | + |
| 19 | M | 69 | FAD | + | ++ | 0 | +++ |
| 20 | M | 71 | FAD | + | ++ | 0 | +++ |
| 21 | M | 71 | AD | ++ | ++ | +++ | ++ |
| 22 | F | 80 | AD | ++ | +++ | + | ++ |
| 23 | F | 80 | AD | + | +++ | +++ | +++ |
| 24 | M | 81 | AD | ++ | ++ | + | + |
| 25 | M | 86 | AD | ++ | ++ | + | ++ |
| 26 | F | 87 | AD | +++ | ++ | ++ | + |
| 27 | F | 88 | AD | +++ | +++ | 0 | 0 |
| 28 | F | 95 | AD | ++ | +++ | 0 | ++ |

M= Male; F = female; C = control case; BACE = immunostaining with the polyclonal antibody goat anti-BACE; SP = senile plaques immunostained with the monoclonal antibody mouse anti-Aβ; NFT = neurofibrillary tangles stained with the Gallyas method; GFAP = immunostaining with the polyclonal antibody rabbit anti-human glial fibrillary acidic protein. Semiquantitative estimation data, taking into account both the intensity of staining and the number of stained elements, were graded as 0 = no staining; + = weak; ++ = medium; +++ = strong; ++++ = very strong.

**Fig. 2.** Box-plot representation of BACE + profiles, Aβ + SP, NFT and GFAP + reactive astrocytes estimations. For data analysis, semiquantitative estimation data from tables 2 and 3, taking into account both the intensity of staining and the number of stained elements, were graded from 0 (no staining) to 4 (++++ = very strong staining). Within the box-plot, the box itself represents the interquartile range (inferior quartile range = 25%; superior quartile range = 75%), the horizontal line, the median value and the cross, the mean; the vertical lines (whiskers) indicate values laying outside the box within a range of ×1.5 that of interquartiles and the square symbol represents values out of the whisker range. There are no significant differences between control (C) and AD cases for BACE + profiles in any of the regions, while significant differences are observed in all regions except DG for SP and NFT. Differences for GFAP + reactive astrocytes are significant in all regions except DG and CA3–4. Between regions, the difference in BACE expression is significant only for CA3–4 versus others in both AD and controls. For probabilities, see text.




3




4

**Table 4.** Mean (± SD) number of cellular profiles per square millimeter of cortex in 7-μm sections

| | | Neurons BACE+ | Neurons BACE– | Total neurons | Astrocytes BACE+ |
|---|---|---|---|---|---|
| Entorhinal cortex | C | 98.4 ± 27.4 | 38.4 ± 9.8 | 136.9 ± 35.7 | 9.9 ± 4.7 |
| | AD | 82.5 ± 10.1 | 16.3 ± 17.7 | 98.7 ± 25.0 | 74.7 ± 112.6 |
| Frontal cortex (area 10) | C | 182.2 ± 37.6 | 81.8 ± 20.1 | 264.0 ± 47.4 | 7.1 ± 6.2 |
| | AD | 188.5 ± 51.7 | 104.2 ± 50.0 | 292.8 ± 83.4 | 9.8 ± 8.0 |

C = Control cases. In the EC, the difference between AD patients and controls is statistically significant only for the number of BACE-negative neuronal profiles (p = 0.037) and for the number of BACE-positive astrocytes (p = 0.005). In the FC, there are no significant differences.

(fig. 4A–D). Thus, neurons expressing BACE do degenerate in the course of AD, or in other words, degenerating neurons still express intracytoplasmic BACE. Finally, regarding the semiquantitative estimation of GFAP staining, Kruskal-Wallis analysis indicated that in all regions except DG and CA3–4, GFAP expression was significantly higher in AD patients than in controls (EC: p = 0.007; SUB: p = 0.003; CA1: p = 0.041; FC: p = 0.001).

We tested the possible correlation between BACE immunostaining and the degree of NFT degeneration or SP deposition in pooled aged controls and AD patients, using a nonparametric Spearman correlation. No significant positive correlation was found between neuronal BACE expression and the amount of amyloid SP, NFT or GFAP staining. A significant positive correlation between the amount of SP and NFT degeneration was found in all regions except DG (EC: R = 0.63, p = 0.021; SUB: R = 0.079, p = 0.001; CA1: R = 0.77, p = 0.002; CA3–4: R = 0.89, $p < 0.001$; FC: R = 0.81, $p < 0.001$).

*Quantitative Analysis*

For quantitative analysis, only EC and FC10 have been taken into account. The number of BACE-positive (strong staining) and BACE-negative (no or very weak staining) neuronal profiles were calculated per square millimeter (see Materials and Methods) and similar data were obtained for BACE-positive reactive astrocytes (table 4). Variations between controls and AD patients were analyzed with a Kruskal-Wallis test. In EC, we observed lower neuronal values in AD patients compared to controls. However, the difference was statistically significant only for BACE-negative neuronal profiles (p = 0.037), not for BACE-positive neuronal profiles. The total number of profiles was decreased, but the difference was at the limit of significance (p = 0.057). In FC, the densities of BACE-positive, BACE-negative and total neuronal profiles were almost similar and differences were not statistically significant. An interesting feature was the presence of a very high number of BACE-positive reactive astrocytes in EC of AD cases, about 7 times more than in controls (p = 0.005), but this was not the case in FC10 (see Discussion). Densities for BACE-positive profiles were also analyzed separately at three different cortical levels corresponding roughly to superficial, medium and deep layers, but Kruskal-Wallis analysis indicated no significant difference between AD patients and controls in the different layers (data not shown). However, there was a significant difference in the surface area frequency distribution of BACE-positive neu-

**Fig. 3.** Photomicrographs of 7-μm paraffin sections showing SP in one 84-year-old control (case 12, EC, layer 2; table 1). **A** SP is marked with anti-BACE antibody revealed with DAB; the center is not marked. **B** SP is marked with anti-BACE antibody revealed with Novared, combined with anti-Aβ revealed with the nickel method; the center is obviously Aβ positive, while the periphery indicates both BACE and Aβ positivity; in addition, an astrocyte is clearly BACE positive. **C** SP marked with anti-GFAP antibody revealed with Novared, combined with anti-Aβ revealed with the nickel method; again the center is obviously Aβ positive, while the periphery indicates GFAP staining and a reactive positive astrocyte. All sections were counterstained with hematoxylin and Scott blue. Scale = 50 μm.

**Fig. 4.** Photomicrographs of 7-μm paraffin sections showing BACE staining in NFT following a two-step protocol. **A**, **C** SUB marked with anti-BACE antibody revealed with DAB in case 22 (table 1). **B**, **D** SUB stained with Gallyas and light green counterstaining on the same section. **A**, **B** Scale: 100 μm. **C**, **D** Scale: 25 μm. Neurons marked with an arrow or asterisk demonstrate clearly that BACE staining is present together with NFT degeneration; the neuron with an asterisk is enlarged in **C** and **D** and shows that BACE immunocytochemical staining seems to parallel NFT degeneration fibrils.

ronal profiles between AD and control cases. A $\chi^2$ test demonstrated more small neuronal profiles than expected in AD compared to controls in the superficial, medium and deep layers of both EC and FC10 ($p < 0.001$).

## Discussion

Our data have shown that the immunoexpression of the β-secretase enzyme, BACE, is not increased in vulnerable brain neurons of the entorhinohippocampal regions of AD brains, or in pyramidal neurons of the FC. In addition, BACE expression is strong in CA3–4 pyramidal neurons and present to a lesser degree in granular neurons of the DG, less vulnerable to the neurodegeneration process. Our semiquantitative analysis indicated that in all regions examined, the level of expression was similar for control and AD cases, while the only statistically significant difference was between regions, with a higher BACE expression in CA3–4 for both control and AD groups. However, data in the EC reached the limit of significance and we performed a quantitative analysis of BACE-positive neuronal profiles in EC compared to FC10. Although the estimated mean number of neuronal BACE-positive profiles per square millimeter of cortex was lower in EC of AD cases compared to controls, the results were not statistically significant and confirmed the semiquantitative analysis. As the mean values for BACE-negative and total numbers of profiles were also decreased, this phenomenon is probably due to neuronal loss, which has been clearly demonstrated in AD EC [24]. Degeneration of BACE neuronal profiles was further attested in our data, by their smaller surface area in AD compared to controls and by the localization of BACE in NFT degenerating neurons marked by Gallyas argentation. Another study established that the density of BACE immunoreactivity was significantly decreased in EC of AD cases, while it was not changed in CA4 and SUB, and increased only in CA1 [19], partially in agreement with our estimations. But to our knowledge, our study is the first showing unchanged numbers of BACE neuronal profiles per square millimeter of cortex in EC and FC. Quantification of BACE mRNA showed no significant change in hippocampal sections of AD brains [15, 16], suggesting an equally stable level of protein, although a recent paper indicated some increase [14]. Other studies showed increased BACE expression in AD cerebral cortex by Western blots [13, 14] and/or increased BACE enzymatic activity in the temporal cortex or other regions [14, 17, 18], as well as in normal aging of several species [25]. However, these studies do not distinguish between neuronal BACE profiles and BACE expression in and around SP or in reactive astrocytes. While the EC begins to loose neurons in early AD [24], the temporal cortex appears damaged with progressing AD [26]. Therefore, a raise in BACE enzymatic activity may be explained by increased activity in surviving neurons, or by a glial upregulation, which has not been studied by the authors [17]. In our data, the number of BACE-positive reactive astrocytes in EC has been found highly increased in AD, thus influencing the overall immunoreactivity.

Altered processing of APP is considered a key event in the pathological cascade of AD and BACE is critical in Aβ biosynthesis, leading to amyloid deposits and SP. BACE has been shown in almost all cellular compartments where APP is found [27], with a maximal activity located within the lumen of acidic intracellular compartments [11]. This may favor the catalytic activity required for Aβ formation and suggests a positive correlation between the enzymatic expression of BACE and Aβ deposit [14, 25]. We observed no positive correlation between the immunocytochemical expression of BACE and Aβ deposit in our cases, but this does not contradict a possible raise in enzymatic activity. Other authors have shown that if BACE activity increased with age, the BACE protein level was unchanged [25]. Interestingly, among the examined structures, the CA3–4 subdivision of the hippocampus has the strongest BACE expression and shows little or no SP and NFT, in spite of many reactive astrocytes. This implies the existence of other functions for BACE than APP processing in humans, in agreement with observations showing that BACE had probably other physiological substrates in cell lines [28]. In FC pyramidal neurons, BACE is also present, without concomitant severe degeneration. Similar BACE staining in intact brain regions was reported by the authors cited above, questioning the role of BACE in healthy neurons.

Altogether, our results suggest that the BACE neuronal level is not the limiting factor in the pathological cascade leading to Aβ deposit in humans. This appears similar in mice, where no differences in BACE expression were observed between normal and transgenic lines overexpressing APP [29–32]. On the other hand, transgenic mice expressing human BACE showed increased amyloidogenic processing of APP, as demonstrated by increased levels of Aβ40 and Aβ42, suggesting that the rate-limiting cleavage in the generation of Aβ was regulated by BACE [32]. Besides, BACE knockout mice have been healthy [12] and BACE knockout mice overexpressing Swedish APP had no Aβ in the brain, leading to the proposal of a therapeutic

BACE inhibition, aimed to reduce Aβ in the brain of AD patients [11]. Recently, double transgenic mice, BACE knockout and overexpressing human APP, showed behavioral and electrophysiological rescue correlated with a dramatic decrease in Aβ deposit [33], further validating BACE inhibition as a therapeutic target. As normal astrocytes exhibit little β-secretase activity [11], the presence of BACE in reactive AD astrocytes – also marked by GFAP – constitutes an intriguing phenomenon, already observed in aged APP transgenic mice [31, 34, 35]. In double transgenic mice, overexpressing both BACE and its substrate APP, BACE-reactive astrocytes were associated with an accelerated amyloid plaque formation [35]. In our data, positive astrocytes were often in the vicinity of Aβ SP, mainly in the entorhinohippocampal region, suggesting a direct link between SP formation and induction of BACE expression in reactive astrocytes in human. Indeed, under chronic but not acute experimental stress, astrocytes, contrarily to microglial cells, appear to express BACE, which simulates Alzheimer long-term degeneration [34]. A critical step in neurodegeneration seems to be reached when pathological glial activation includes astrocytes in addition to microglia [36]: differentiated reactive astrocytes no longer appear to be able to control microglial nitric oxide production, leading to the production of neurotoxic peroxynitrates, or to maintain extracellular ion homeostase, favoring excitotoxicity. The whole neuronal microenvironment may be troubled, contributing to oxidative stress, the latter being able in turn to increase BACE neuronal production or activity [37].

Finally, the exact mechanism leading to neurodegeneration in sporadic AD is still unknown. Within the dominant conceptual framework of the Aβ cascade, one should certainly reconsider the competition between β-secretase amyloidogenic and α-secretase nonamyloidogenic cleavage [38]. A pathway involving sumoylation regulating α-mediated versus β-mediated cleavage of APP has been described [39] and puts emphasis on targeting the stimulation of α-secretase as a possible drug to reduce Aβ. So far, the expression of α-secretase has not been considered predominant in the cascade. But a decrease in α-secretase activity has been shown in the temporal cortex of AD patients without concomitant decrease in immunocytochemical expression [18], while a curious increase in α-secretase mRNA has been shown in AD hippocampus and cerebellum, without relationship to the severity of damage [15]. Yet, modulation of the competition between α- and β-secretase activities for the APP substrate has to be studied in greater details, as in both mice and humans they are colocalized in cortical neurons, together with APP [40]. The total Aβ deposit, however, largely depends on Aβ clearance and degradation, under the influence of factors such as ApoE, $\alpha_2$-macroglobulin or insulin-degrading enzyme among others [41]. Against the view of Aβ as a major causative factor in AD, several papers have described Aβ deposits in the brain of old controls without dementia [42, 43], but others seem to indicate a correlation between Aβ deposit and cognitive decline [44, 45]. Further, it has been shown that immunization is able to clear Aβ from the brain of AD patients [46] and that immunized patients generating antibodies against Aβ exhibit a slower cognitive decline [47]. Other authors see the production of Aβ peptide, Aβ40 particularly, as a critical requirement for the viability of central neurons, as the presence of Aβ prevented the toxicity of β- and γ-secretase inhibitors on neuronal cell cultures [48]. In addition, APP processing seems to be involved in synaptic function, with endogenous Aβ participating in a feedback control of neuronal activity [49]. Therefore, in spite of already existing statin-based peptidomimetic inhibitors of β-secretase [50, 51], the modulation of endogenous Aβ under the control of secretases should be foreseen with some caution with regard to possible known and unknown side effects. Our study shows that the level of BACE protein expression is not necessarily linked to Aβ formation but is increased in activated astrocytes from AD brains. Further, strong BACE expression in healthy neurons suggests alternate unknown functions.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